Predigestion of Soybean Proteins with Immobilized Trypsin for Infant Formula

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ABSTRACT

Soybean protein isolates of low soybean trypsin inhibitor (STI) residue were prepared by acidic precipitation of soybean flour water extracts (0.8–1.2%) at pH 5.0, followed by acidic washing at this pH and affinity adsorption of residual STI with immobilized trypsin on polystyrene anion-exchange resin GM 201. After heat treatment, soybean protein isolates were subjected to controlled hydrolysis with the immobilized trypsin. Then, the predigested soybean protein was prepared. The predigested soybean protein was free of STI activity, and its solubility at acidic pH range was greatly increased. Sedimentation test showed that it formed a much finer clot at pH 4.5 than that of untreated soybean protein. The pepsin digestibility index at pH 4.0 and chymotrypsin digestibility index at pH 8.0 were obviously improved. These results suggested that the predigested soybean protein prepared by this method may be used in infant formulas.

Index Entries: Soybean protein; immobilized trypsin; protein digestibility; protein enzymatic modification; infant formula.

INTRODUCTION

Soybean is an important source of dietary protein in China. It is of nutritional significance with a biological value of 62.5, which is close to that of bovine casein, whose biological value is 73. However, the value of food protein is related not only to its nutritional value, but also to the digestibility and other factors of antinutrients. The digestibility of soybean proteins is relatively poor because the main component of proteins

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is soybean globulins, which are compact in structure and difficult to digest. In addition, high levels of trypsin inhibitor activity in soybean are also of nutritional concern (*1,2*). Animal feeding tests have shown that raw soybean flour and purified soybean trypsin inhibitor decrease growth and affect the pancreas. These factors have limited the use of soybean proteins in infant formulas.

In order to improve the digestibility of soybean proteins and to eliminate the deleterious effect of soybean trypsin inhibitor, we have developed a method for preparing soybean protein isolates by affinity chromatography and have investigated the effect of limited hydrolysis by immobilized trypsin to improve the digestibility of soybean protein isolates so that the modified product could be used in infant formulas.

MATERIALS AND METHODS

Materials and Reagents

Defatted soybean flour was a product of Sanjiang Food Products Co., Heilongjiang, China. Polystyrene anion-exchange resin GM 201 was a product of the Chemical Factory of Nankai University, Tianjin. The resin was first treated with 70% alcohol, 1 mol/L NaOH, 0.5 mol/L $(NH_4)_2SO_4$, and 3 mol/L HCl, and then used for trypsin immobilization. Pepsin and chymotrypsin were from Shanghai Biochemicals Co. Sephadex G-15, 25, 50, and 75 were products of Pharmacia. Other reagents were analytical grade chemicals.

Immobilized Trypsin

Trypsin was prepared from fresh swine pancreatin with the method of Van Melle et al. (*3*). The pretreated anion-exchange resin GM 201 was used as matrix for enzyme immobilization. The procedure for trypsin immobilization and enzyme activity determination of the immobilized trypsin was conducted with previously reported methods (*1*).

Trypsin Inhibitor Activity of Soybean Protein

The trypsin inhibitor activity of soybean protein was determined by the method of Hamerstrand et al. (*2*).

Protein Determination

Protein was assayed by the method of Lowry (*4*).

Reactors for Protein Digestion with Immobilized Trypsin

Two reactor types were used in the experiments of controlled hydrolysis: the batch stirred reactor (BSR) and the packed column bed reactor (PCBR, 35 × 400 mm, packed with 150 g of immobilized trypsin with specific activity (SA) of 600 μ/g). Soybean protein isolates after heat treatment were solubilized at pH 8.0 and kept at 45°C for 30 min before hydrolysis. When BSR was used, digestion was begun with the addition of the immobilized trypsin (10–20 g for 100 mL protein solution), and the degree of hydrolysis was controlled by reaction time. At the end of the reaction, immobilized trypsin was separated from the reaction mixture by filtration. When PCBR was used, hydrolysis was controlled by adjusting the flow rate of soybean protein solution. For both types of reactors, the temperature of reaction was maintained at 45°C.

Predigestion Ratio of Enzymatic Modified Soybean Proteins

The predigestion ratio (PDR) was determined by the following method. One milliliter of predigested soybean protein solution was added to 9 mL 0.2 mol/L acetate buffer, pH 4.5. The mixture was stirred vigorously, and allowed to stand at room temperature for 30 min and then centrifuged. The total protein of the predigested sample and the protein content of supernatant (mainly oligopeptides) were determined by the Lowry method. PDR was defined as follows:

$$\text{PDR} = (\text{protein content of supernatant} / \text{total protein in predigested sample}) \times 100\% \quad (1)$$

Sedimentation Curves of Predigested Soybean Proteins

The isoelectric precipitate sedimentation curve of the predigested soybean protein solutions was determined by adding 18 mL 0.2 mol/L acetate buffer, pH 4.5, to sample of 2 mL of predigested soybean protein solution in a 20-mL graduated test tube. After stirring vigorously, the precipitate was allowed to settle, and the precipitate level and the time elapsed were recorded.

Digestibility Index of the Predigested Soybean Proteins

The pepsin digestibility index and chymotrypsin digestibility were determined according to the methods of Akeson and Stahann (*5*) and

Li-Chan and Nakai (*6*) with slight modification. The modified procedure was as follows. Predigested soybean protein (100 mL) (5 mg/mL) was adjusted to a certain pH value and kept at 37°C for at least 30 min. Then, the appropriate amount of pepsin or chymotrypsin was added, mixed, and kept at 37°C for 30 min. Solubility of protein of the reaction mixture in 5% trichloric acid solution was determined and defined as the digestibility index of this predigested soybean protein sample.

RESULTS AND DISCUSSIONS

Preparation of Soybean Protein Isolates of Low Trypsin Inhibitor Activity by Affinity Chromatography

Soybean has relatively high levels of trypsin inhibitors (TI) and has received special attention in food processing. The residual soybean trypsin inhibitors activity (STIA) of protein isolates prepared from water extracts of defatted soybean flour by acid precipitation varied from 20–30%. Acid precipitation with the addition of 0.1 mol/L NaCl reduced the residual TIA to a level of 5–7% (*7*).

We have developed a method for the preparation of soybean protein isolates practically devoid of TIA by affinity chromatography on immobilized trypsin (*1*). Polystyrene anion-exchange resin GM 201, which had been purified to remove impurities, was first treated with glutaraldehyde, and then coupled with swine pancreatic trypsin of SA 5,000 μ/mg (with benzoylarginine *p*-nitroanilide, BAPA as substrate) at 4°C, pH 8.0. The immobilized trypsin thus obtained had good heat stability and operational stability as shown in Fig. 1 and Table 1, respectively.

The starting material for our preparations was commercial defatted soybean flour. Defatted soybean flour was extracted with water at pH 9.0, and the extract was diluted with water to a protein concentration of 0.8–1.2% and then precipitated with acid at pH 5.0. The precipitates, mainly soybean globulins, were washed with water (pH 5.0) twice, and the washings were added to the wheys. Resolubilization with water at pH 9.0 and precipitation with acid at pH 5.0 were repeated twice. The resulting precipitates and wheys were analyzed for trypsin inhibitor activity (*2*) and for proteins. It was found that the trypsin inhibitor content of soybean protein isolates was reduced to 1.85%, whereas the specific activity dropped to <1 μg/mg. The yield of soybean globulins was 62%.

To remove the trypsin inhibitor from soybean protein isolates further, affinity chromatography on the immobilized trypsin was used. Activity of the immobilized trypsin was 570 μ/g (BAPA). The affinity column (10 × 120 mm) was first equilibrated with 0.01 mol/L borate buffer, pH 7.8, containing 0.3 mol/L NaCl and 0.01 mol/L $CaCl_2$ and water. Soybean

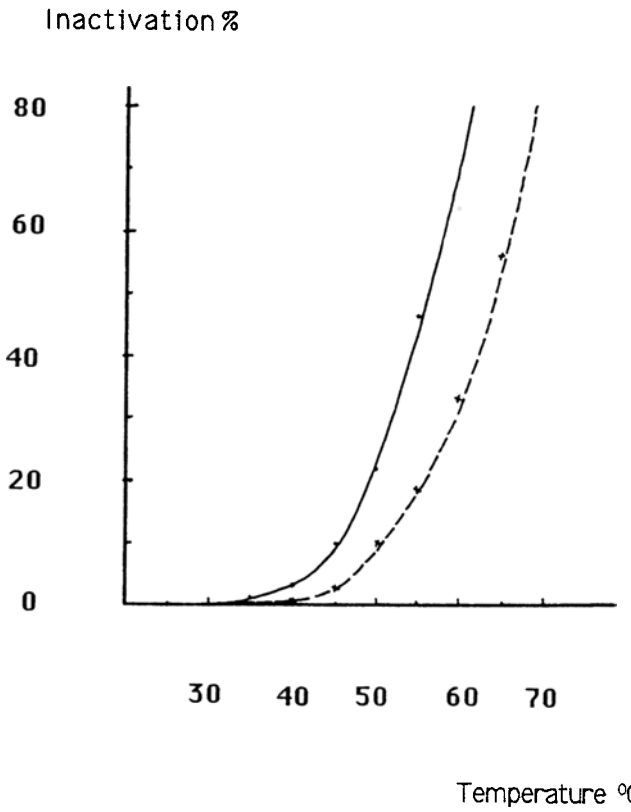

Fig. 1. Heat inactivation of free (——) and immobilized (------) trypsin with casein as substrate. Trypsin solution and immobilized trypsin were heated in water bath for 30 min at a certain temperature, and then activity was determined.

Table 1
Operational Stability of the Immobilized Trypsin

Batch no.	1	2	3	4
Product conc. mg/mL[a]	0.32	0.36	0.42	0.42
TIA adsorbed (μ/g)[b]	836	860	832	-

[a]Casein hydrolyates (50 mL 0.5% casein solution hydrolyzed with 5 g immobilized trypsin, 50°C, 10 min).
[b]STI adsorbed (100 mL soybean whey treated with 10 g immobilized trypsin).

protein isolates obtained from the above were dissolved in water, pH 7.8, and loaded onto the affinity column. Then, the column was washed with the same borate buffer. The eluents and the washings were combined and analyzed for trypsin inhibitor activity and proteins. The results of affinity chromatography showed that the soybean protein isolates thus obtained were practically devoid of STIA (Table 2).

Table 2
Low STI Residue Soybean Protein Preparation

Procedural steps[a]	Soybean trypsin inhibitor activity			Soybean protein	
	μ/mL	μ/mg	%	mg/mL	%
1	1160	29.36	100	39.5	100
2	81.3	2.71	7.08	30	75.95
3	43.5	1.50	3.83	29	73.42
4	21.49	0.877	1.85	24.5	62.03
5	0	0	0	24.5	62.03

[a]1. Water extraction of soybean flour at pH 9.0.
2. Precipitating the water extracts at pH 5.0, and then washed at this pH.
3. Solubilization of the precipitate, followed by precipitation at pH 5.0.
4. Repeat operation 3.
5. Solubilization of the precipitate and then affinity chromatography.

The affinity column was washed with water and with 0.1 mol/L borate buffer, pH 7.8, containing 0.3 mol/L NaCl and 0.01 mol/L $CaCl_2$. Then it was regenerated finally by eluting with 0.25 mol/L KCl-HCl buffer, pH 2.0, to wash out the STI adsorbed on the affinity column. After equilibrating with pH 7.8 borate buffer, the column could be reused.

Controlled Hydrolysis of Soybean Protein Isolates of Low Trypsin Inhibitor Activity by Immobilized Trypsin

Soybean protein isolates of low STIA prepared by affinity chromatography on immobilized trypsin column was used as the starting material for controlled hydrolysis. It was found that the STIA of the product obtained from affinity chromatography slowly increased after a period of storage. This may be due to the existence of an aggregated form of soybean trypsin inhibitor that prevented it from being absorbed by immobilized trypsin. In order to inactivate the possible aggregated soybean trypsin inhibitor and to facilitate enzymatic hydrolysis, soybean protein isolates after affinity chromatography were subjected to heat treatment at 100°C for 20–30 min to denature the proteins prior to controlled hydrolysis. After treatment with the immobilized trypsin, samples of predigested soybean protein solutions were subjected to various analyses.

Factors Affecting the PDR of Soybean Protein Isolates

In order to optimize the experimental conditions for controlled hydrolysis, factors that affected the PDR of soybean protein isolates were investigated. As mentioned before, heat treatment at 100°C for 20–30 min

not only inactivated the trypsin inhibitor, but also increased the PDR of soybean protein isolates by a factor of about 1.2 because of heat denaturation of soybean proteins.

When soybean protein solutions of different concentrations were allowed to react with immobilized trypsin, it could be found that the PDR reached optimum when protein concentration was about 10 mg/mL. If protein concentration increased further beyond this optimum concentration, the PDR progressively decreased. This may be due to the low efficiency of mass transfer at high concentration of proteins.

The PDR of soybean protein isolates was also affected when different types of reactors were used for enzymic hydrolysis. When BSR at 45°C was used, hydrolysis was relatively slow. In addition, the chance of getting contaminated was increased with prolonged periods of hydrolysis. When PCBR was used, the rate of hydrolysis was considerably faster, and hydrolysis could be carried out continuously by passing the soybean protein solutions through packed columns connected in a series.

Composition and Properties of Predigested Soybean Proteins

After treatment with immobilized trypsin, the soybean protein solutions were adjusted to pH 4.5 and allowed to stand for 30 min. Then they were centrifuged. The supernatant consisted of oligopeptides that were soluble at pH 4.5, whereas the residue consisted of modified soybean globulins that were not soluble at this pH. Both products were subjected to gel filtration on Sephadex G-75, G-50, G-25, and G-15 (1 × 120 cm) to determine their molecular size distribution. The eluent used was 0.1 mol/L phosphate buffer, pH 7.5, containing 0.05 mol/L NaCl. When a solution of modified soybean globulins was placed on a Sephadex G-75 column, it passed down the column more slowly than untreated soybean globulins, and only a single peak appeared in gel filtration chromatogram. Similar results were obtained with Sephadex G-50 and G-25 columns. However, when Sephadex G-15 was used for gel filtration of the supernatant solution, three peaks appeared in the chromatogram (Fig. 2), indicating the molecular size of the oligopeptides was smaller than 1500.

The physicochemical properties of the predigested soybean proteins changed considerably after treatment with immobilized trypsin. The sedimentation velocity of predigested soybean proteins at pH 4.5 was much slower, and the precipitate was much finer than that of untreated soybean proteins as shown in the isoelectric precipitation curves (Fig. 3). It could be seen that the higher the PDR, the finer the precipitate and the slower the sedimentation velocity. Acidic clotting of proteins is the first step of digestion in human stomach. When proteins are precipitated in a very fine state, this will exert a favorable effect on peptic digestion.

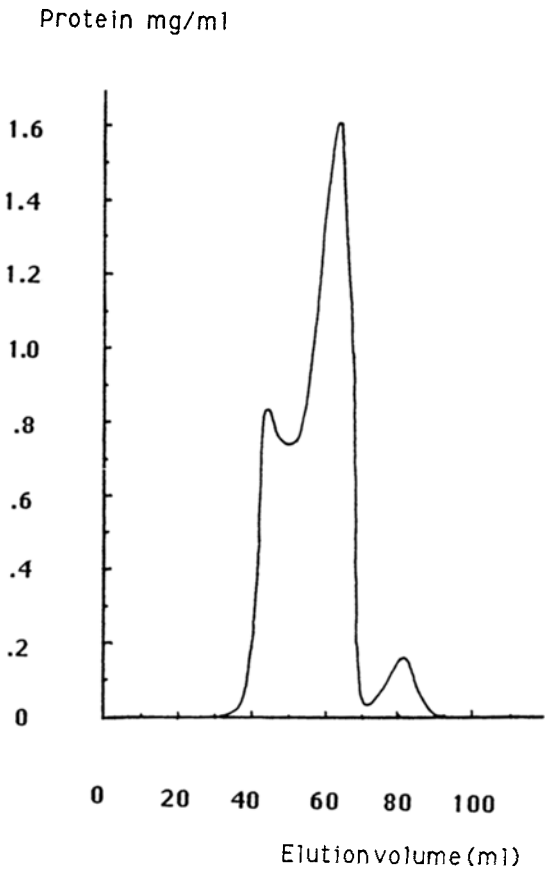

Fig. 2. Elution profiles of isoelectric soluble oligopeptides from predigested soybean protein isolates on Sephadex G-15 (V_0 = 34 mL).

The solubility of predigested soybean protein isolates in the range of pH 4.0–6.0 was higher than that of untreated soybean protein isolates. It could be seen from Fig. 4 that the higher the PDR, the more the solubility increased. At very high PDR, the solubility of predigested soybean protein isolates was practically independent of pH changes.

Digestibility of Predigested Soybean Proteins

In order to evaluate the digestibility of soybean proteins after treatment with immobilized trypsin, we compared the pepsin digestibility index and chymotrypsin digestibility index of untreated soybean protein isolates, predigested soybean protein isolates, and modified soybean globulins. The pepsin digestibility index at pH 4.0 of predigested soybean protein isolates was higher than that of untreated samples as shown in Table 4, and the higher the PDR, the higher the pepsin digestibility index. However,

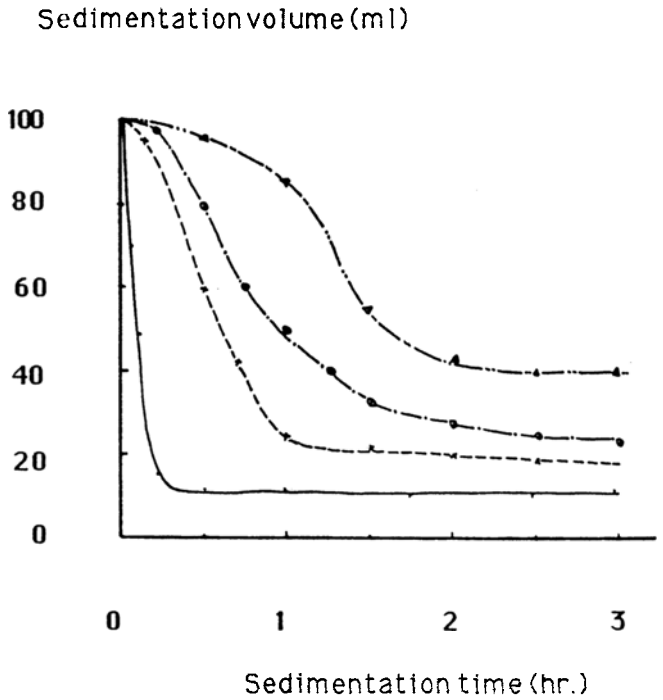

Fig. 3. Sedimentation behavior of the predigested soybean protein isolates at pH 4.5. ——— PDR = 0, ----- PDR = 10.60%, -·-·- PDR = 15.56%, -·-·-·- PDR = 18.35%.

the pepsin digestibility index at pH 2.0 of predigested soybean protein isolates was somewhat lower than that of untreated samples. A similar phenomenon was also observed in the case of casein predigested with rennet (*6*). The chymotrypsin digestibility index of predigested soybean protein isolates at pH 8.0 was higher than that of untreated samples (Table 3), and the higher the PDR, the higher the chymotrypsin digestibility index.

During the process of digestion in human stomach, the soluble oligopeptides present in predigested soybean protein isolates will soon pass out from the stomach, but the modified soybean globulins will remain in the stomach to be digested. We therefore compared the pepsin digestibility index and the chymotrypsin digestibility index of modified soybean globulins and soybean globulins obtained from acid precipitation without treatment with immobilized trypsin. The pepsin digestibility index at pH 2.0 and 4.0 and chymotrypsin digestibility index at pH 8.0 of the modified soybean globulins were increased (Table 4). The higher the PDR, the higher the pepsin and chymotrypsin digestibility index.

All these results suggested that controlled hydrolysis of soybean protein isolates by immobilized trypsin improved the digestibility of soybean proteins. Since the predigested soybean protein is easily digestible and

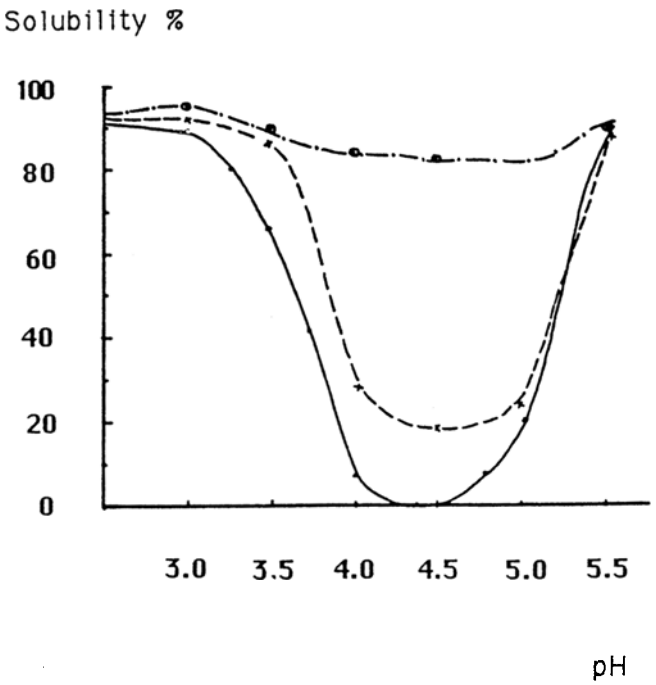

Fig. 4. Relationship between pH and solutibility of predigested soybean protein isolates. ——— PDR = 0, ----- PDR = 23%, ·---· PDR = 80%.

Table 3
Effects of Predigestion Ratio (PDR)
on the Digestibility of Soybean Protein Isolates

PDR, %	Pepsin digestibility index		Chymotrypsin digestibility index, pH 8.0
	pH 2.0	pH 4.0	
0	.6850	.3116	.2587
10.60	.6600	.3381	.4586
15.56	.6145	.3834	.5155
16.85	.5684	.4429	.5625
18.35	.5517	.3956	.5704
79.50	.5337	.6350	.6820

the immobilized trypsin was considered safe for the food industry (*1*), the predigested soybean protein may be used as protein extender in cereal-based milk substitute or as an ingredient for infant formulas.

SUMMARY

A new method for the preparation of soybean protein isolates with low soybean trypsin inhibitor activity was developed that included pre-

Table 4
Effects of Predigestion Ratio (PDR)
on the Digestibility of Modified Soybean Globulins

PDR, %	Pepsin digestibility index		Chymotrypsin digestibility index, pH 8.0
	pH 2.0	pH 4.0	
0	.8134	.2587	.1176
10.60	.8330	.3352	.3234
15.56	.8546	.3254	.4116
16.85	.7664	.3626	.4253
18.35	.8996	.3880	.4645

liminary separation of trypsin inhibitor from aqueous extract (pH 9.0) of commercial defatted soybean flour by acidic precipitation (pH 5.0), followed by affinity chromatography on immobilized trypsin. Soybean protein isolates with low trypsin inhibitor activity after controlled hydrolysis by immobilized trypsin could be separated by isoelectric precipitation (pH 4.5) into two fractions: soluble, easily assimilable oligopeptides with molecular size below 1500 and tryptic-modified soybean globulins. The pepsin digestibility index (pH 4.0) and chymotrypsin digestibility index (pH 8.0) of soybean protein isolates and tryptic-modified soybean globulins were increased with increase of predigestion ratio. These results suggested that controlled hydrolysis by immobilized trypsin improves the digestibility of soybean proteins, and thus, the present methods may be used to prepare predigested soybean proteins for infant formulas.

REFERENCES

1. Ge, S. J. and Zhang, L. X. (1991), *Chinese Biochem. J.* **7,** 339.
2. Hamerstrand, G. E., Black, L. T., and Glover, J. D. (1981), *Cereal Chem.* **58,** 42.
3. Van Melle, P. J., Lewis, S. H., Samsu, E. G. et al. (1963), *Enzymologia* **26,** 133.
4. Lowry, O. H., Rosbrough, N. J., Farr, A. L. et al. (1951), *J. Biol. Chem.* **193,** 265.
5. Akeson, W. R. and Stahann, M. A. (1964), *J. Nutrition* **83,** 257.
6. Li-Chan, E. and Nakai, S. (1988), *Can. Inst. Food Sci. Technol. J.* **21,** 200.
7. Honig, D. H., Rackis, J. J., and Wolf, W. J. (1987), *J. Agric. Food Chem.* **35,** 967.